IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Hadlaczky et al.	Art Unit	: 1638
Serial No.	: 09/724,726	Examiner	: Helmer, G.L.
Filed	: November 28, 2000	Cust No.	: 20985
Conf. No.	: 7776		
Title	: *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*		

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Gyula Hadlaczky declare as follows:

1) I am a co-inventor of the above-identified U.S. Patent Application Serial No. 09/724,726.

2) I have read the accompanying Declaration of Dr. Steven F. Fabijanski, which demonstrates the generation of SATACs in plants using the methods as taught in the application. This Declaration is incorporated herein by reference.

3) I am currently a Professor and Scientific Advisor at the Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences in Szeged, Hungary. I have held these positions since April 1993. Prior to that time, I held the positions of Senior Researcher (March 1983 – April 1993), Researcher (June 1980 – March 1983), and Assistant Researcher (February 1973 – June 1980) all at the Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences. I also am a cofounder of Chromos Molecular, Inc., a now-public Canadian company, which was founded to exploit the satellite artificial chromosomes (designated SATACs in the application; and presently referred to as Aces) described in the application. Chromos Molecular, Inc. has successfully commercialized the SATACs, including the spin-out of a subsidiary directed to plant applications of SATACs.

4) I received a Certificated Engineer degree from the University of Agricultural Sciences, Gödöllô, Hungary in 1972. I received University Doctor degree in cytogenetics from the University of Agricultural Sciences, Gödöllô, Hungary in 1974, and a Ph.D. degree in experimental biology from the Hungarian Academy of Sciences, Hungary in 1978. I

received D.Sci. in Biology from the Hungarian Academy of Sciences, Hungary in 1993. I have over 35 years experience working in the fields of chromosome and artificial chromosome technology, including the generation and uses of artificial chromosomes. I have authored or co-authored over 65 publications, and I have presented over 100 lectures in these fields. I also have received numerous awards and honors for my Scientific achievements including the distinction of Honorary Professor of the Department of Biotechnology at Gödöllô University; recipient of the Research Award of the Hungarian Academy of Sciences in 1974; recipient of the Academic Award for Young Scientists of the Hungarian Academy of Sciences in 1976; and recipient of the "Straub F. Bruno" Award from the Biological Research Center of the Hungarian Academy of Sciences in 1991. I also was the recipient of the Széchenyi award in 2000 for the work that is the subject of the above-captioned application.

5) The above-captioned application describes the generation of and preparation of satellite artificial chromosomes (designated SATACs in the application; and presently referred to as Aces). As provided in the application, this method is generally applicable to eukaryotic species to produce SATACs in any such species. The process by which SATACs are generated is a universal process, fundamental to replication and recombination in cells. As described in the above-captioned application, introduction of nucleic acid into the heterochromatic pericentric region of the chromosome, whether by targeted introduction or random introduction, initiates amplification events, leading to the generation of the *de novo* centromere, and ultimately of a SATAC. The underlying process of generating SATACs, including an amplification event and generation of a *de novo* centromere, is based on a universal mechanism shared by all species and cell types. This is described in the above-captioned application. As evidence of the universality of the process, this Declaration, and the accompanying Declaration of Fabijanski, describe the generation of SATACs in such diverse species as mammals, including rodents and human, and also in plants.

6) Using methods and materials described in the above-referenced application, and standard methods as described herein, myself and other scientists involved in these projects have demonstrated the generation of plant and human SATACs by the introduction of heterologous DNA into plant or human/hamster hybrid cells, respectively, leading to the amplification event that results in the generation of SATACs. This Declaration, and the accompanying declaration of Steven F. Fabijanski demonstrate the generation of SATACs in human/hamster hybrid cells and plants, respectively. The SATACS are generated as taught in the specification. Analysis of the resulting plant and human SATACs show that, as

taught in the application, plant and human satellite artificial chromosomes share the same identifying characteristics as mouse satellite artificial chromosomes, which are specifically exemplified in the subject application. Hence the methods used to generate SATACs in rodents is reproducible and can be applied to other species and also in other host cells. Therefore, as described in the above-captioned application, the underlying processes that result in preparation of SATACs are universally shared among species. Based upon the teachings in this application, SATACs can be generated in any selected species.

A description of the above-referenced methods and results follows.

I. MATERIALS AND METHODS

Generation of Human Artificial Chromosomes (Human SATACs)

1. Transfection of the 94.3 Cell line with Foreign DNA

a. The 94.3 Cell Line

A human/rodent somatic cell hybrid cell line, referred to as 94-3 (Repository No. GM10664), was obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository (Camden, NJ). This cell line was generated by PEG fusion of human lymphoblasts with the HPRT-deficient Chinese Hamster cell line RJK88. The hybrid cells contain a translocated human chromosome 15 (the human der(15)t(X;15)) and human chromosome 22. The cells were cultured in DMEM medium containing 10% FBS.

The presence of human chromosomes in 94-3 cells was confirmed by *in situ* hybridization using biotin-labeled human genomic DNA probes and biotin-labeled human alpha satellite DNA probes. The hybridized probes were detected with FITC-conjugated avidin and with biotinylated anti-avidin. The chromosomes were counterstained with propidium iodide. The results of this analysis revealed that greater than 80% of the cells carried one or two human chromosomes.

b. Transfection of 94-3 Cells

Semiconfluent dishes (5 x 10^6 cells) of 94-3 cells were cotransfected with a mixture of ~1 μg pBabe Puro, ~2 μg pCH110 and ~70 μg pK161 using a calcium phosphate DNA precipitation method (Pharmacia).

i. pBabe Puro

pBabe Puro is a bacterial plasmid construct that contains DNA encoding the ampicillin resistance gene, the pUC origin of replication, and the puromycin N-acetyl transferase gene under control of the SV40 early promoter.

ii. pCH110

Plasmid pCH110 (Pharmacia) contains a β-galactosidase gene.

iii. pK161

Plasmid pK161 was generated by ligation of a 9-kb coding sequence of a mouse ribosomal RNA-encoding gene (*i.e.*, rDNA) with the cosmid vector pWE15 (Stratagene, La Jolla, California). The rDNA was obtained from megachromosomes that had been isolated from 1B3 cells (which were generated by repeated BrdU treatment and single cell cloning of H1xHE41 cells as described in the above-captioned application on page 30, lines 2-9, and page 71, lines 14-25, and which contain a truncated megachromosome) using fluorescence-activated cell sorting methods as described in the above-referenced application. The 9-kb rDNA fragment was isolated as follows. Following separation of the megachromosomes from the endogenous chromosomes in 1B3 cells, the isolated megachromosomes were stored in GH buffer (100 mM glycine, 1% hexylene glycol, pH 8.4-8.6 adjusted with saturated calcium hydroxide solution) and centrifuged into an agarose bed in 0.5 M EDTA. The isolated megachromosomes were cleaved with *Not*I, a rare cutting restriction endonuclease with an 8-bp GC recognition site. Fragments of the megachromosome were inserted into plasmid pWE15 (Stratagene, La Jolla, California) as follows. Half of a 100-µl low melting point agarose block (mega-plug) containing the isolated megachromosomes was digested with *Not*I overnight at 37°C. Plasmid pWE15 was similarly digested with *Not*I overnight. The mega-plug was then melted and mixed with the digested plasmid, ligation buffer and T4 ligase. Ligation was conducted at 16°C overnight. Bacterial DH5α cells were transformed with the ligation product and transformed cells were plated onto LB/Amp plates. Fifteen to twenty colonies were grown on each plate for a total of 189 colonies. Plasmid DNA was isolated from colonies that survived growth on LB/Amp medium and was analyzed by Southern blot hybridization for the presence of DNA that hybridized to a pUC19 probe. This screening methodology assured that all clones, even clones lacking an insert but containing the pWE15 plasmid, would be detected. All colonies were positive for hybridizing DNA.

Liquid cultures of all 189 transformants were used to generate cosmid minipreps for analysis of restriction sites within the insert DNA. Six of the original 189 cosmid clones contained an insert. One of these clones was designated pK161 (~9-kb insert). Portions of the sequence of the insert in pK161 were determined using an ABI sequencer and the dye-terminator cycle protocol.

A comparison of the sequence data to sequences in the GENBANK database revealed that the insert of pK161 corresponds to an internal section of the mouse ribosomal RNA gene (rDNA) repeat unit between positions 7551-15670 as set forth in GENBANK accession no. X82564. The sequence of the insert in pK161 diverges in some positions from the sequence presented in positions 7551-15670 of GENBANK accession no. X82564. Such divergence may be attributable to random mutations between repeat units of rDNA.

2. Analysis of Transfectants

Forty-eight hours after transfection of 94-3 cells with foreign DNA, the cells were exposed to 10μg/ml puromycin (Sigma). Sixty-eight individual puromycin-resistant colonies were selected and propagated and then analyzed for the presence of artificial chromosomes by Southern hybridization, LacZ staining, C-banding and *in situ* hybridization.

II. Results

The results demonstrate that human artificial chromosomes (human SATACs) can be generated in human cells.

Generation of a Human SATAC

1. Selection and Screening for Amplification

Southern hybridization of DNA isolated from the selected 94-3 cell transfectants was used as a primary screen to detect cells containing chromosomes that had undergone amplification of the pericentric DNA indicative of satellite artificial chromosome formation. DNA purified from the transfectants was digested with EcoRI and hybridized with pBabe Puro. DNA from more than 40% of the 68 selected transfectants showed hybridization at levels indicative of high-copy numbers of integrated pBabe Puro sequences. This result correlates with an amplification of the integrated pBabe Puro DNA. Transfectants were also analyzed for expression of β-galactosidase from the integrated pCH110 DNA by using standard LacZ-staining techniques. Twenty-one of the 68 transfectants showed β-galactosidase expression detectable in this assay.

Amplification also was assessed by cytological analysis of the transfectant by C-banding according to the Giemsa/barium hydroxide method (see *e.g.*, Sumner (1972) *Exp. Cell Res.*, 75:304-306). This staining method specifically detects constitutive heterochromatin. Thirty percent of the transfectants showed amplified heterochromatic segments through C-banding. Because Chinese hamster cell chromosomes do not contain any large constitutive heterochromatic regions, whereas human chromosomes 15 and 22 do have somewhat larger regions of constitutive heterochromatin, the presence of chromosomes

containing extensive regions that are readily detectable by C-banding supports the conclusion that amplification of the heterochromatin had most likely occurred in the human chromosomes of many of the transfectants.

2. Detection and Characterization of Artificial Chromosomes

a. C-banding

One of the transfected clones (clone 23) was analyzed further. C-banding of clone 23 revealed the presence of a sausage chromosome with the characteristic extended heterochromatic arm. A human satellite artificial chromosome that is ~100-120 Mb in size also was detected in clone 23. C-banding of the chromosomes in clone 23 cells revealed that the human satellite artificial chromosome resulted from further amplification of the heterochromatic arm of a sausage chromosome. Both arms of the human artificial chromosome stained as predominantly heterochromatic, in contrast to a sausage chromosome in which only one arm stains in C-banding. The presence of two heterochromatic arms indicates that a sausage chromosome had undergone amplification, including a centromere duplication, leading to the formation of another heterochromatic chromosome arm and the generation of a satellite artificial chromosome.

b. *In Situ* Hybridization

Clone 23 also was subjected to *in situ* hybridization with corresponding propidium iodide staining to counterstain the chromosome structure. Hybridization with a biotinylated probe containing human genomic DNA from the EJ30 cell line revealed that the site of hybridization of the probe was on the chromosome containing the heterochromatic arm (*i.e.* sausage chromosome). Thus, it was possible to conclude that the heterochromatic arm was formed on a human chromosome. Further analysis with a biotinylated human alpha satellite DNA probe confirmed that the heterochromatic arm was formed on a human chromosome, and *in situ* hybridization with a biotin-labeled human chromosome 15-specific alpha satellite DNA probe (Oncor catalog no. P5034 D15Z1) revealed that the saugage chromosome derived from human chromosome 15. *In situ* hybridization of clone 23 with pBabe Puro and rDNA (contained in pK161) probes revealed a hybridization signal only in the heterochromatic arm of the sausage chromosome, indicating that these genes co-amplified in the heterochromatic arm of the sausage chromsome.

Analysis of clone 23 cells by hybridization to biotin-labeled human alpha satellite DNA and biotin-labeled pBabe Puro also was used in characterization of the human satellite artificial chromosome. Because the hybridization of the two different probes was to the same chromosome, these results demonstrate that the artificial chromosome contains the intergrated foreign DNA and is human in origin. Additionally, *in situ* hybridization of clone 23 cells with a biotin-labeled human chromosome 15-specific alpha satellite DNA probe (Oncor catalog no. P5034 D15Z1) revealed that the satellite artificial chromosome derived from human chromosome 15.

III. CONCLUSION

The above experiments demonstrate that the SATAC's are generated by a fundamental and universal process. This is evidenced by the above data, and the data in the accompanying Declaration of Fabijanski. The data demonstrate the preparation of SATACs in such diverse species as human and plant cells. The results show that the generation of a plant or human SATAC is induced by the integration of exogenous DNA molecules into the pericentric regions of the native chromosome, which leads to a large-scale amplification and *de novo* formation of a stable, heterochromatic SATAC. Thus, the inducible replication-directed large scale amplification and *de novo* chromosome formations are general across diverse species.

I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeapardize the validity of the application or any patent resulting therefrom.

Date

Gyula Hadlaczky

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Gyula Hadlaczky et al.	Art Unit	: 1638
Serial No.	: 09/724,726	Examiner	: Helmer, Georgia L.
Filed	: November 28, 2000	Conf. No.	: 7776
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P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL LETTER

Dear Sir:

Transmitted herewith are a Preliminary Amendment and Request for Continued Examination (RCE) pursuant to 37 C.F.R. §1.114 responsive to the Office Action mailed March 30, 2006 and the Notice of Appeal filed September 29,2006, Unsigned Declarations of Gyula Hadlaczky and Steven Fabijanski, a Supplemental Information Disclosure Statement, Form PTO-1449 (1 page), copies of three foreign Office Actions, copies of four U.S. Office Actions, cited non U.S. patent documents listed on the form PTO-1449, documents listed in table of Supplementary Information Disclosure Statement (12 documents), a check ($1475.00) for the requisite fee for the RCE filing fee and a five-month extension of time fee, an and a return postcard in connection with the above-captioned patent application. If a Petition for extension of time is needed, this paper is to be considered such Petition.

Extension fee for a five-month extension of time and RCE fee:

☒ By a small entity.....$1080.00 + $395.00=$1475.00

☒ The Commissioner is hereby authorized to charge the fee for the extension of time and any other fee that may be due in connection with this and the attached papers or with this application during its entire pendency to Deposit Account No. 06-1050. A duplicate of this sheet is enclosed.

Respectfully submitted,

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I hereby certify that this paper is being deposited with the United States Postal "Express Mail Post Office to Addressee" Service under 37 CFR §1.10 on the date indicated above and is addressed to: Commissioner for Patents, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA, 22313-1450.

Stephanie Seidman

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Steven F. Fabijanski declare as follows:

1) I am familiar with the subject matter of the above-captioned application, which was filed on November 28, 2000, as well as the parent applications, including the earliest application U.S. Application Serial No. 08/629,822.

2) I have reviewed the Office Action, mailed March 30, 2006, in connection with the above-captioned application.

3) I received a Bachelor's degree in Biology from the University of Miami (Florida) in 1977. I received a Ph.D. degree in Cellular and Molecular Biology from the University of Southern California in 1981. I have held post-doctoral positions at the University of Ottawa in Ottawa, Ontario, Canada and the University of Southern California in Los Angeles, Califonia from 1982 to 1985. From 1986 to 1991, I held the position of Research Director at Paladin Hybrids, Inc.

4) I have over 20 years of experience in the area of plant molecular biology, plant gene expression, plant tissue and cell culture and development of techniques to produce genetically modified plants and plant artificial chromosomes. I have authored or co-authored over 20 publications and I am an inventor of over 16 US and foreign patents.

5) I am currently Director of Research and Development at Agrisoma Biosciences Inc., located in 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9. Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9, is an owner of Agrisoma Biosciences, Inc., to whom the subject matter of this application has been licensed.

6) I have held the position of Director since 2001. I am also President of the FAAR Biotechnology Group, Inc., located at Suite 323, 5929L Jeanne D'arc Boulevard, Orleans, Ontario, Canada K1C 7K2. I have held this position since 1992.

7) The above-captioned application describes the generation of satallite artificial chromosomes (designated SATACs in the application, and presently referred to as ACes), including plant SATACs. As described in the above-captioned application, SATACs, including plant SATACs, are generated following introduction of nucleic acid into the heterochromatic pericentric region of the chromosome, whether by targeted introduction or random introduction, which initiates amplification events leading to the generation of the *de novo* centromere, and ultimately of a SATAC. For example, as described in the above-captioned application at page 6, line 15 to page 7, line 23, SATACs are generated by introduction of heterologous nucleic acid encoding one or more selectable markers into cells, growing cells, and identifying from the resulting clones those that include chromosomes with more than one centromere and/or fragments thereof. If desired, the heterologous nucleic acid includes sequences that target it to an amplifiable region, such as the pericentric region, of heterochromatin. Those of skill in the art, in working with conventional cytological techniques, have long described that herterochromatin can include the nucleolar organizer region comprising rDNA and have further described that rDNA found in the nucleolar organizing region can be identified within pericentric heterochromatin by the use of FISH, for example (see e.g., Hizume, M., *et al.*(1992) *Jpn. J. Genet.* 67: 389-396). Many different plant species are known to have rDNA sequences found within pericentric heterochromatin on acrocentric chromosomes (see e.g., Sato, S., *et al.* (1980) *Cytologia* 45: 87-96; Maluszynska J and Heslop-Harrison JS (1991) *Plant J.* 1: 159–166). In this Declaration, FISH images showing the pericentric localization of rDNA, in particular rDNA localized to the short arms of acrocentric chromosomes found in *Nicotiana* and *Brassica* plant species, is included.

8) In my capacity as researcher and Director of Research, I have personally supervised experiments related to the production of plant SATACs from two distinct plant species: *Nicotiana* and *Brassica*. *Nicotiana* is a species of plant from the core eudicots group, namely the Solanales within the euasterids I group of the asterids clase, which encompass nearly one-third of all angiosperm species. *Brassica* is a species of plant (a cruciferous plant) from the eurosids II group of the rosid clade, which encompass close to one-third of all angiosperm species. Numerous SATACs, and also precursors and

intermediates, such as dicentric chromosomes and sausage chromosomes, in the formation of SATACs have been generated in these plant species. Thus, plant SATACs have been successfully generated in distantly related plant species.

9) Using methods and materials described in the above-referenced application, and standard methods as described herein, myself and other scientists involved in these projects have generated multiple plant SATACs by introduction of DNA into plant cells as taught in the application. Analysis of the transfected plant cells, identified the presence of plant SATACs, and precursors and intermediates in the formation of plant SATACs including "sausage" chromosomes and dicentric chromosomes.

Hence, as described in the application, SATACs can be generated by producing cells that contain dicentric or multicentric chromosomes; and then culturing cells. When cultured, dicentric and multicentric chromosomes break to form a minichromosome and a formerly dicentric (or multicentric) chromosome, which after amplification events results in a SATAC. These methods described in the application have been employed to generate SATACs in plants as described in the application. As described in the application and based on the teachings of the specification, no knowledge of centromere DNA sequences is required to generate a SATAC in any species, including a plant SATAC.

Also, the results of this work demonstrate that a plant satellite artificial chromosome generated following the teachings in the above-captioned application possess the structural, physical and functional characteristics of a plant satellite artificial chromosome (*i.e.* a plant SATAC) described in the above-captioned application.

A description of the above-referenced methods and and the resulting production of several different SATACs in two different plant species is described in the following sections.

I. MATERIALS AND METHODS

A. Generation of Plant SATACs in *Nicotiana*

1. Construction of Heterologous DNA

a. Selectable Marker DNA

The nucleic acid sequence encoding a selectable marker was constructed by routine recombinant techniques by joining the Arabidopsis polyubiqutin 10 (UBQ10) 5' and 3' flanking regions (Norris *et al.* (1993) *Plant Mol Biol.*, 21:895-906) to the phosphinothricin N-acetyltransferase (PAT) gene (Wohlleben W *et al.* (1988) *Gene*, 70:25-37), carried in a pBluescript backbone (Stratagene, La Jolla, CA). This resulted in a selectable marker

construct conferring constitutive resistance to the herbicidal compound L-phosphinothricin (L-PPT) referred to as pDAB 2416. The nucleotide sequence of this vector is shown in Exhibit A.

b. Targeting DNA to the pericentric region

A targeting DNA molecule was constructed to target the integration of the selectable marker to the pericentric region, in particular the pericentric rDNA, of an acrocentric chromosome. The coding region of the 26S rDNA was chosen as a targeting sequence because it is highly conserved among species and it encodes a structural RNA molecule highly conserved among eukaryotic organisms. There are multiple sources of rDNA coding sequences available, dating back as early as 1985 (see *e.g.*, Takaiwa *et al.* (1985) Gene, 37:255-9.)

The coding region of a plant 26S rDNA (approximately 1.7 Kb) was chosen and isolated from the Arabidopsis rDNA repeat using published sequence and restriction mapping information (Genbank Accession no. X52320, which was deposited in the early 1990s; see also Pruit and Meyerowitz (1991) J Mol Biol., 187:169-83; Genbank Accession no. X15550; Gruender et al. (1991) J Mol Biol. 221:1209-1222). The targeting DNA was cloned into vector pBluescript (Stratagene, La Jolla, CA).

2. Introduction of DNAs into plant cells, selection and identification of amplified DNA

DNA encoding the selectable marker and targeting DNA were introduced into *Nicotiana* protoplasts using standard methods. Using standard methods, the DNA fragment encoding the selectable marker, comprised in the pDAB 2416 construct as shown in Exhibit A, was gel purified and introduced along with targeting DNA comprising the coding sequence of the 26S rDNA. In these experiments, both the selectable marker and targeting DNA were introduced into plant cells free of vector backbone, and were introduced into plant cells using PEG mediated transfection.

To illustrate the general method, tobacco cells were used. Briefly, tobacco protoplasts were isolated from established sterile tobacco plant cultures by immersion of sterile tissue in enzyme solution containing 1.2% Cellulase 'Onozuka' R-10 and 0.4% Macerozyme R-10. The protoplasts were purified by pouring through a 100 um nylon mesh sieve, overlayed with washing solution and centrifuged at 80xg for 10 min. Protoplasts were then resuspended at a density of $1x10^6$ protoplasts/ml and stored at 4°C for 1 to 2 hours prior to DNA uptake. Plasmid DNAs from the vector and targeting DNA were sterilized with

chloroform and 70% ethanol before use for transfection. A protoplast suspension was mixed with vector and targeting DNA at a ratio of 1:10 followed immediately by slowly adding PEG solution. Typically 30 μg of DNA mixture (vector and targetting DNA at a 1:10 ratio) were used per 1 X 10^6 protoplasts. The mixture was incubated at 22°C for 10-15 minutes with gentle shaking. The protoplasts were resuspended and cultured at 22°C in the dark. When microcalli developed, the protoplasts were embedded in 0.6% agarose.

Selection on protoplast cultures was carried out by adding L-PPT to the medium at a final concentration of 10 mg/l, 14 to 21 days after transfection. Following selection, plant cells were analyzed at the molecular level by southern blot for the presence of the selectable marker. Plant cells also were analyzed at the cytological level for co-localization of the selectable marker to pericentric DNA, amplification of pericentric DNA and for the presence of SATACs. For example, two-color fluorescent in situ hybridization (FISH) was performed using two probes. The first probe recognized the selectable marker sequence and was visualized with a fluorescein isothiocyanate (FITC) tag (yellow-green fluorescence). The second probe was tagged with rhodamine (red fluorescence) and recognized pericentric DNA (18S or 26S rDNA) sequences endogenous to *Nicotiana* cells.

To obtain spreads of metaphase chromosomes, cells were subjected to either a single blocking protocol (colchicine treatment) or double blocking protocol (for example, treating plant cells with 5 mg/l aphidocolin for 24 hours and then 1.54 mg/ml Propyzamide for 4 hours.) Blocked cells were recovered and chromosome spreads prepared and subjected to two-color FISH. Red and yellow-green fluorescence was monitored to identify amplification. The chromosome spreads were analyzed for the presence of structural features of chromosomes.

B. Generation of Plant SATACs in *Brassica napus*

The methods for generating plant SATACs are not unique to any singular selectable marker or plant species. Using methods as set forth in the above-referenced application, and as set forth above for the generation of SATACs in *Nicotiana*, heterologous DNA also was introduced into *Brassica napus* to produce plant SATACs, following selection and amplification of pericentric DNA.

The heterologous DNA included a construct containing a CaMv 35S promoter fused to a phosphinothricin acetyl transferase gene (*bar)* as a selectable marker (White *et al.* (1989) *Nucleic Acids Res.*, 18:1062), with an *att B* recombination site between the 35S promoter and *bar* selection gene, and was contained in a pBluescript backbone (Stratagene, La Jolla, CA),

referred to as pABI 012. The nucleotide sequence of this selectable marker is shown in Exhibit B. The DNA encoding the *bar* selectable marker was introduced along with 26S targeting DNA, as described above for generation of plant SATACs in *Nicotiana.* Typically 1-10 X 10^6 mesophyl protoplasts were isolated and used for DNA uptake. *Brassica napus* protoplasts were isolated from mesophyll material derived from *in vitro* cultured shoots essentially as described by Vamling K. and Glimelius K. (see e.g., Legumes and Oilseed Crops I in Biotechnology in Agriculture and Forestry 10, Springer Verlag (1990)). As done with *Nicotiana* species, plasmid DNAs from the vector and targeting DNA were sterilized with chloroform and 70% ethanol before use for transfection. A protoplast suspension was mixed with vector and targeting DNA at a ratio of 1:10 followed immediately by slowly adding PEG solution. Typically, 30 μg of DNA mixture (vector and targeting DNA at a 1:10 ratio) were used per 1 X 10^6 protoplasts.

Selection was on L-PPT and the plants were regenerated used standard protocols known in the art, *e.g.* those described in: Vamling K. and Glimelius K. (see *e.g.*, Legumes and Oilseed Crops I in Biotechnology in Agriculture and Forestry 10, Springer Verlag (1990); Glimelius K. *et al.* (1986) *Plant Sci.* 45, 133 – 144; Barsby *et al.* (1986) *Plant Cell Reports 5*, 101). Typically, up to 50% of L-PPT resistant calli regenerated to shoots and whole plants. The resultant spreads of chromosomes were analyzed by two-color FISH for the presence of chromosome structures as described above for *Nicotiana*, and in the above-referenced application.

II. Results

A. *Nicotiana*

The results show that by using the methods as described in the specification in plant cells, chromosomes were generated having the characteristic structural features (e.g., arms and a centromeric region) of chromosomes based on the description of satellite artificial chromosome generation in the above-captioned application and parent application U.S. Application Serial No. 08/629,822. These results provide evidence that homologous recombination between the exogenous DNAs and rDNA of the *Nicotiana* chromosomes had occurred, and that large scale amplification of pericentric DNA resulted. For example, a comparison of the yellow-green signal (to detect the selectable marker) and red staining (to detect 18S rDNA) of the same chromosome spread revealed overlap of the signals. Areas where significant levels of both yellow-green and red signals were observed demonstrate

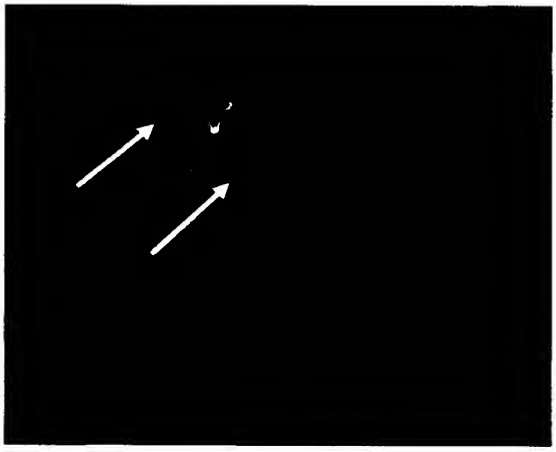
large-scale amplification of the pericentric DNA. This includes production of "sausage" chromosomes and amplification of heterochromatin satellite DNA, such as pericentric rDNA.

Over 20 independent SATACs or precursors and intermediates in the formation of SATACs (i.e. dicentric chromosomes or sausage chromosomes) have been generated in *Nicotiana*. Some exemplary chromosome structures are described below.

1. Dicentric chromosome

Figure 1 depicts a cell line whereby insertion of vector DNA into the pericentric heterochromatic DNA (rDNA) resulted in formation of a dicentric chromosome. As there are no centromere specific probes available for *Nicotiana*, a cytological observation by FISH analysis of centromere constriction was used to identify dicentric chromosomes. The chromosome in Figure 1 was stained with DAPI (blue) and co-stained for the presence of the selectable marker (FITC: yellow-green) and pericentric heterochromatic DNA (Rhodamine:red). The results show that the chromosome containing the amplified pericentric DNA contains two regions that show typical condensation and constriction indicative of a centromere region.

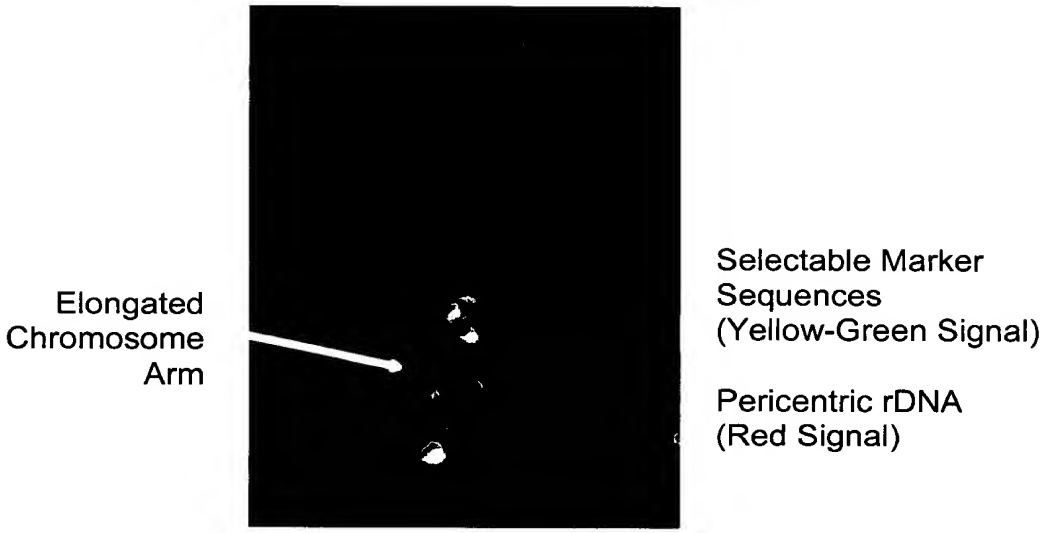
Figure 1: Dicentric Chromosome



2. Sausage Chromosome

Figure 2 depicts cytological analysis demonstrating the presence of a "sausage" chromosome generated in *Nicotiana* according to the method described herein, and in the above-referenced application. The chromosome in Figure 2 was stained with DAPI (blue) and co-stained for the presence of the selectable marker (FITC: yellow-green) and pericentric heterochromatic DNA (Rhodamine:red). The elongated chromosome arm depicted in Figure 2 demonstrates a periodicity of signal consistent with the "sausage" chromosome.

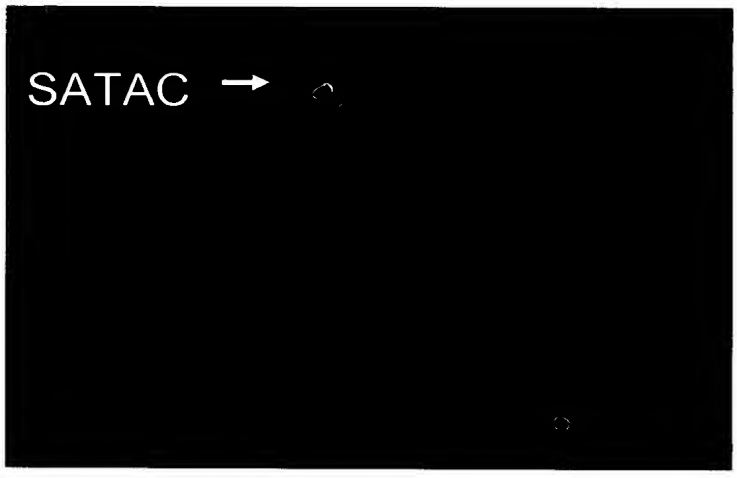
Figure 2: Sausage Chromosome



3. SATAC

Figure 3 depicts the results of cytological analysis evidencing the presence of a typical SATAC generated in *Nicotiana* according to the method described herein. FISH analysis demonstrates the presence of a chromosome containing multiple copies of the selectable marker (FITC: yellow-green) and a substantial amount of amplified pericentric heterochromatic DNA (Rhodamine: red) on a distinct chromosome structure. This evidences the generation of a SATAC.

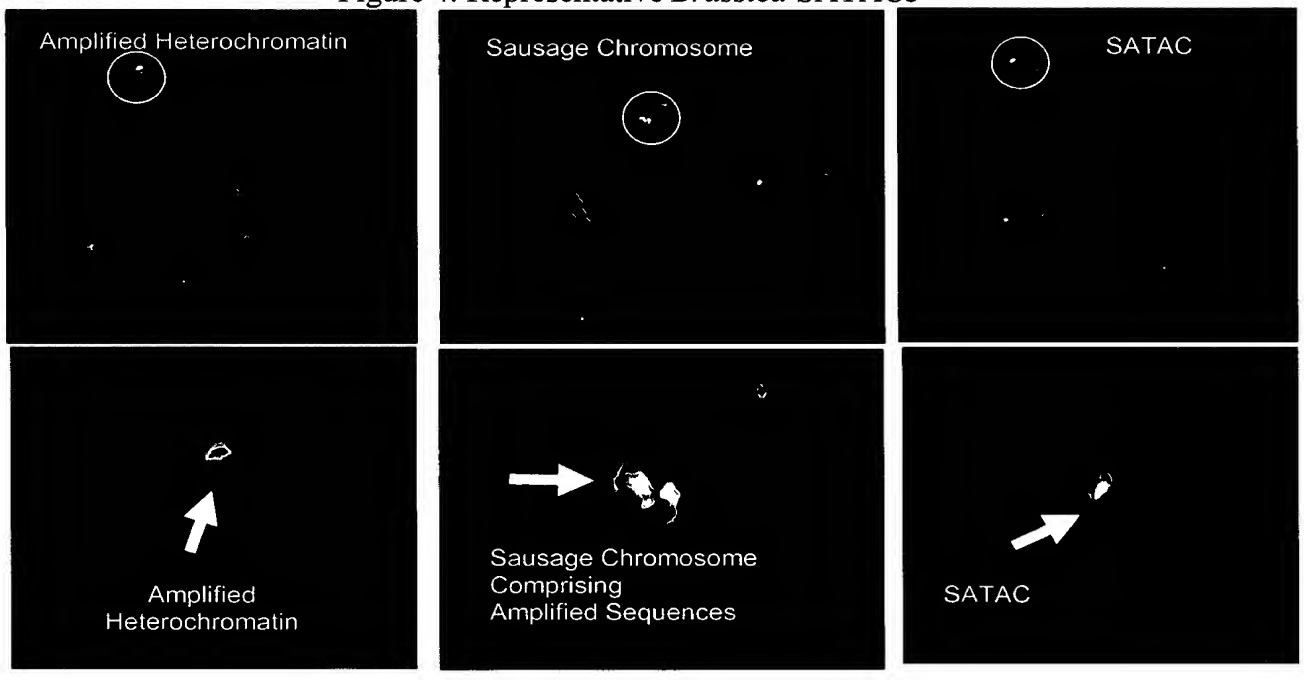
Figure 3: Typical SATAC



B. *Brassica napus*

Plant SATACs also were generated in *Brassica napus*. Figure 4 shows the results of FISH analysis of a root tip spread from a *Brassica* plant following introduction of heterologous DNA under selective conditions. The results in Figure 4 show that amplified selectable marker sequences are within amplified pericentric DNA evidencing homologous recombination between the exogenous DNAs and rDNA of the *Brassica* chromosomes. Southern blot analysis indicated that the selectable marker is represented at greater than 20 copies within this structure. Multiple structures of chromosomes having identifying characteristics as described in the above-referenced application for the generation of SATACs, were observed in *Brassica,* following selection and regeneration to whole plants. More than 10 SATACs and precursors and intermediates in the formation of SATACs have been generated and identified in *Brassica*. Some exemplary chromosome structures are depicted in Figure 4. As above, the chromosome spreads were stained with DAPI (blue) and co-stained for the presence of the selectable marker (FITC: yellow-green) and pericentric heterochromatic DNA (Rhodamine: red).

Figure 4: Representative *Brassica* SATACs



III. Conclusion

The results of the experiments provided herein demonstrate that by following the teachings of the specification and employing standard methods as described herein, plant satellite artificial chromosomes can be generated and selected within plants. The teachings of the specification provide a means to target a selectable marker to pericentric heterochromatin and to recover chromosomes that have amplified pericentric DNA, including dicentric or multicentric SATACs. The nature of the DNA sequences that can be employed are not limited to any particular DNA molecule. Further, the results herein depict that the method is broadly applicable to all plant species, including the distinct species of plants of *Nicotiana* and *Brassica.* Accordingly, the experiments described herein demonstrate the generation of satellite artificial chromosomes in plants using methods as taught in the application, resulting in chromosomes having the same identifying characteristics and structural features as taught in the application.

I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeapardize the validity of the application or any patent resulting therefrom.

_____ _____

Date Steven F. Fabijanski

Exhibit A: Annotated Nucleotide Sequence of pDAB 2416 Selectable Marker Vector

```
   1  GTCGACCTGC AGGTCAACGG ATCAGGATAT TCTTGTTTAA GATGTTGAAC TCTATGGAGG TTTGTATGAA CTGATGATCT AGGACCGGAT AAGTTCCCTT CTTCATAGCG
      >>.....................................................AtUbi10 Pro v2.....................................................>

 111  AACTTATTCA AAGAATGTTT TGTGTATCAT TCTTGTTACA TTGTTATTAA TGAAAAAATA TTATTGGTCA TTGGACTGAA CACGAGTGTT AAATATGGAC CAGGCCCCAA
      >.....................................................AtUbi10 Pro v2.....................................................>

 221  ATAAGATCCA TTGATATATG AATTAAATAA CAAGAATAAA TCGAGTCACC AAACCACTTG CCTTTTTTAA CGAGACTTGT TCACCAACTT GATACAAAAG TCATTATCCT
      >.....................................................AtUbi10 Pro v2.....................................................>

 331  ATGCAAATCA ATAATCATAC AAAAATATCC AATAACACTA AAAAATTAAA AGAAATGGAT AATTTCACAA TATGTTATAC GATAAAGAAG TTACTTTTCC AAGAAATTCA
      >.....................................................AtUbi10 Pro v2.....................................................>

 441  CTGATTTTAT AAGCCCACTT GCATTAGATA AATGGCAAAA AAAAACAAAA AGGAAAAGAA ATAAAGCACG AAGAATTCTA GAAAATACGA AATACGCTTC AATGCAGTGG
      >.....................................................AtUbi10 Pro v2.....................................................>

 551  GACCCACGGT TCAATTATTG CCAATTTTCA GCTCCACCGT ATATTTAAAA AATAAAACGA TAATGCTAAA AAAATATAAA TCGTAACGAT CGTTAAATCT CAACGGCTGG
      >.....................................................AtUbi10 Pro v2.....................................................>

 661  ATCTTATGAC GACCGTTAGA AATTGTGGTT GTCGACGAGT CAGTAATAAA CGGCGTCAAA GTGGTTGCAG CCGGCACACA CGAGTCGTGT TTATCAACTC AAAGCACAAA
      >.....................................................AtUbi10 Pro v2.....................................................>

 771  TACTTTTCCT CAACCTAAAA ATAAGGCAAT TAGCCAAAAA CAACTTTGCG TGTAAACAAC GCTCAATACA CGTGTCATTT TATTATTAGC TATTGCTTCA CCGCCTTAGC
      >.....................................................AtUbi10 Pro v2.....................................................>

 881  TTTCTCGTGA CCTAGTCGTC CTCGTCTTTT CTTCTTCTTC TTCTATAAAA CAATACCCAA AGCTTCTTCT TCACAATTCA GATTTCAATT TCTCAAAATC TTAAAAACTT
      >.....................................................AtUbi10 Pro v2.....................................................>

 991  TCTCTCAATT CTCTCTACCG TGATCAAGGT AAATTTCTGT GTTCCTTATT CTCTCAAAAT CTTCGATTTT GTTTTCGTTC GATCCCAATT TCGTATATGT TCTTTGGTTT
      >.....................................................AtUbi10 Pro v2.....................................................>

1101  AGATTCTGTT AATCTTAGAT CGAAGACGAT TTTCTGGGTT TGATCGTTAG ATATCATCTT AATTCTCGAT TAGGGTTTCA TAAATATCAT CCGATTTGTT CAAATAATTT
      >.....................................................AtUbi10 Pro v2.....................................................>

1211  GAGTTTTGTC GAATAATTAC TCTTCGATTT GTGATTTCTA TCTAGATCTG GTGTTAGTTT CTAGTTTGTG CGATCGAATT TGTCGATTAA TCTGAGTTTT TCTGATTAAC
      >.....................................................AtUbi10 Pro v2.....................................................>

1321  AGGTAAGGAT CCAACCATGG CTTCTCCGGA GAGGAGACCA GTTGAGATTA GGCCAGCTAC AGCAGCTGAT ATGGCCGCGG TTTGTGATAT CGTTAACCAT TACATTGAGA
      >> AtUbi10 Pro v2
                     >>.....................................................PAT v3.....................................................>

1431  CGTCTACAGT GAACTTTAGG ACAGAGCCAC AAACACCACA AGAGTGGATT GATGATCTAG AGAGGTTGCA AGATAGATAC CCTTGGTTGG TTGCTGAGGT TGAGGGTGTT
      >.....................................................PAT v3.....................................................>

1541  GTGGCTGGTA TTGCTTACGC TGGGCCCTGG AAGGCTAGGA ACGCTTACGA TTGGACAGTT GAGAGTACTG TTTACGTGTC ACATAGGCAT CAAAGGTTGG GCCTAGGATC
      >.....................................................PAT v3.....................................................>
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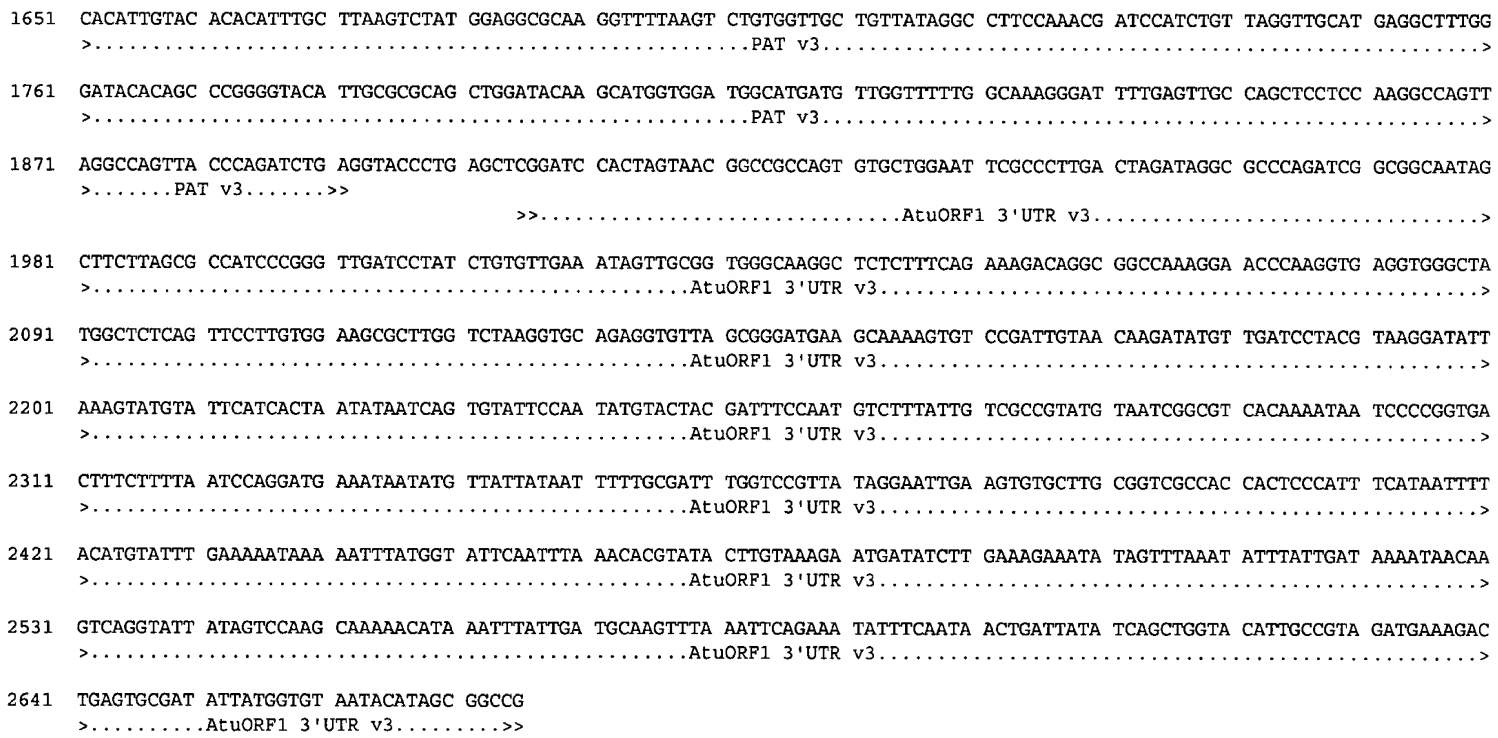
1651 CACATTGTAC ACACATTTGC TTAAGTCTAT GGAGGCGCAA GGTTTTAAGT CTGTGGTTGC TGTTATAGGC CTTCCAAACG ATCCATCTGT TAGGTTGCAT GAGGCTTTGG
>..PAT v3..>
1761 GATACACAGC CCGGGGTACA TTGCGCGCAG CTGGATACAA GCATGGTGGA TGGCATGATG TTGGTTTTTG GCAAAGGGAT TTTGAGTTGC CAGCTCCTCC AAGGCCAGTT
>..PAT v3..>
1871 AGGCCAGTTA CCCAGATCTG AGGTACCCTG AGCTCGGATC CACTAGTAAC GGCCGCCAGT GTGCTGGAAT TCGCCCTTGA CTAGATAGGC GCCCAGATCG GCGGCAATAG
>.....PAT v3.....>>
>>..AtuORF1 3'UTR v3..>
1981 CTTCTTAGCG CCATCCCGGG TTGATCCTAT CTGTGTTGAA ATAGTTGCGG TGGGCAAGGC TCTCTTTCAG AAAGACAGGC GGCCAAAGGA ACCCAAGGTG AGGTGGGCTA
>..AtuORF1 3'UTR v3..>
2091 TGGCTCTCAG TTCCTTGTGG AAGCGCTTGG TCTAAGGTGC AGAGGTGTTA GCGGGATGAA GCAAAAGTGT CCGATTGTAA CAAGATATGT TGATCCTACG TAAGGATATT
>..AtuORF1 3'UTR v3..>
2201 AAAGTATGTA TTCATCACTA ATATAATCAG TGTATTCCAA TATGTACTAC GATTTCCAAT GTCTTTATTG TCGCCGTATG TAATCGGCGT CACAAAATAA TCCCCGGTGA
>..AtuORF1 3'UTR v3..>
2311 CTTTCTTTTA ATCCAGGATG AAATAATATG TTATTATAAT TTTTGCGATT TGGTCCGTTA TAGGAATTGA AGTGTGCTTG CGGTCGCCAC CACTCCCATT TCATAATTTT
>..AtuORF1 3'UTR v3..>
2421 ACATGTATTT GAAAAATAAA AATTTATGGT ATTCAATTTA AACACGTATA CTTGTAAAGA ATGATATCTT GAAAGAAATA TAGTTTAAAT ATTTATTGAT AAAATAACAA
>..AtuORF1 3'UTR v3..>
2531 GTCAGGTATT ATAGTCCAAG CAAAAACATA AATTTATTGA TGCAAGTTTA AATTCAGAAA TATTTCAATA ACTGATTATA TCAGCTGGTA CATTGCCGTA GATGAAAGAC
>..AtuORF1 3'UTR v3..>
2641 TGAGTGCGAT ATTATGGTGT AATACATAGC GGCCG
>.....AtuORF1 3'UTR v3.....>>

Exhibit B: Annotated Nucleotide Sequence of pABI 012 Selectable Marker Vector

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   1  CGACACTCTC GTCTACTCCA AGAATATCAA AGATACAGTC TCAGAAGACC AAAGGGCTAT TGAGACTTTT CAACAAAGGG TAATATCGGG AAACCTCCTC GGATTCCATT
      >>.....................................................CaMV-35S.....................................................>

 111  GCCCAGCTAT CTGTCACTTC ATCAAAAGGA CAGTAGAAAA GGAAGGTGGC ACCTACAAAT GCCATCATTG CGATAAAGGA AAGGCTATCG TTCAAGATGC CTCTGCCGAC
      >.....................................................CaMV-35S.....................................................>

 221  AGTGGTCCCA AAGATGGACC CCCACCCACG AGGAGCATCG TGGAAAAAGA AGACGTTCCA ACCACGTCTT CAAAGCAAGT GGATTGATGT GATAACATGG TGGAGCACGA
      >.....................................................CaMV-35S.....................................................>

 331  CACTCTCGTC TACTCCAAGA ATATCAAAGA TACAGTCTCA GAAGACCAAA GGGCTATTGA GACTTTTCAA CAAAGGGTAA TATCGGGAAA CCTCCTCGGA TTCCATTGCC
      >.....................................................CaMV-35S.....................................................>

 441  CAGCTATCTG TCACTTCATC AAAAGGACAG TAGAAAAGGA AGGTGGCACC TACAAATGCC ATCATTGCGA TAAAGGAAAG GCTATCGTTC AAGATGCCTC TGCCGACAGT
      >.....................................................CaMV-35S.....................................................>

 551  GGTCCCAAAG ATGGACCCCC ACCCACGAGG AGCATCGTGG AAAAAGAAGA CGTTCCAACC ACGTCTTCAA AGCAAGTGGA TTGATGTGAT ATCTCCACTG ACGTAAGGGA
      >.....................................................CaMV-35S.....................................................>

 661  TGACGCACAA TCCCACTATC CTTCGCAAGA CCTTCCTCTA TATAAGGAAG TTCATTTCAT TTGGAGAGGA CACGCTGAAA TCACCAGTCT CTCTCTACAA ATCTATCTCT
      >.....................................................CaMV-35S.....................................................>>
                                                                                                                    Att B <

 771  GTCGAGTGAA GCCTGCTTTT TTATACTAAC TTGAGCGAAC TCGAGTCTAC CATGAGCCCA GAACGACGCC CGGCCGACAT CCGCCGTGCC ACCGAGGCGG ACATGCCGGC
      <.....................Att B...................<<
                                                      >>.............................Bar.............................>

 881  GGTCTGCACC ATCGTCAACC ACTACATCGA GACAAGCACG GTCAACTTCC GTACCGAGCC GCAGGAACCG CAGGAGTGGA CGGACGACCT CGTCCGTCTG CGGGAGCGCT
      >.....................................................Bar.....................................................>

 991  ATCCCTGGCT CGTCGCCGAG GTGGACGGCG AGGTCGCCGG CATCGCCTAC GCGGGCCCCT GGAAGGCACG CAACGCCTAC GACTGGACGG CCGAGTCGAC CGTGTACGTC
      >.....................................................Bar.....................................................>

1101  TCCCCCCGCC ACCAGCGGAC GGGACTGGGC TCCACGCTCT ACACCCACCT GCTGAAGTCC CTGGAGGCAC AGGGCTTCAA GAGCGTGGTC GCTGTCATCG GGCTGCCCAA
      >.....................................................Bar.....................................................>

1211  CGACCCGAGC GTGCGCATGC ACGAGGCGCT CGGATATGCC CCCCGCGGCA TGCTGCGGGC GGCCGGCTTC AAGCACGGGA ACTGGCATGA CGTGGGTTTC TGGCAGCTGG
      >.....................................................Bar.....................................................>

1321  ACTTCAGCCT GCCGGTACCG CCCCGTCCGG TCCTGCCCGT CACCGAGATT TGACTCGAGT TTCTCCATAA TAATGTGTGA GTAGTTCCCA GATAAGGGAA TTAGGGTTCC
      >.........................Bar.........................>>
                                                              >>.....................CaMV polyA.....................>

1431  TATAGGGTTT CGCTCATGTG TTGAGCATAT AAGAAACCCT TAGTATGTAT TTGTATTTGT AAAATACTTC TATCAATAAA ATTTCTAATT CCTAAAACCA AAATCCAGTA
      >.....................................................CaMV polyA.....................................................>
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1541  CTAAAATCCA GATCCCCCGA ATTAATTCGG CGTTAATTCA G
      >.............CaMV polyA.............:...>>
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